(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 555 618 A2**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **92830653.9**

(51) Int. Cl.[5] **A23L 1/03**

(22) Date of filing: **04.12.92**

(30) Priority: **10.02.92 IT MI920256**

(43) Date of publication of application:
18.08.93 Bulletin 93/33

(84) Designated Contracting States:
BE CH DE DK ES FR GB GR IE LI LU NL PT

(71) Applicant: **Cavaliere Vesely, Renata Maria Anna**
Via S. Orsola 11
I-20123 Milano(IT)
Applicant: **De Simone, Claudio**
Via Nuoro 10
I-00040 Ardea (Roma)(IT)

(72) Inventor: **Cavaliere Vesely, Renata Maria Anna**
Via S. Orsola 11
I-20123 Milano(IT)
Inventor: **De Simone, Claudio**
Via Nuoro 10
I-00040 Ardea (Roma)(IT)

(74) Representative: **Righetti, Giuseppe**
Bugnion S.p.A. Via Carlo Farini, 81
I-20159 Milano (IT)

(54) Dietetic and/or pharmaceutical compositions containing lyophilized lactic bacteria, their preparation and use.

(57) Pharmaceutical compositions containing highly-concentrated lyophilized lactic bacteria in combination with pharmacologically acceptable excipients and optionally suitable physiologically compatible drugs, are adapted for restoring the intestinal mucous membrane mass and modulating the epithelial cell kinetics and the enzyme potential of the intestine when they have been altered as a result of stress conditions, and in, addition in case of gastroenteritis, colitis, constipation, diarrhoea, for recolonizing the intestine after food disorders, surgical interventions, chemotherapy or contagious deseases, hepatopathies resulting from toxic, metabolic or infectious causes, as adjuvants for controlling hyperammonemia and endotoxinemia while liver insufficiency is in progress, in case of lack of humoral immunity and of mucosal and systemic cell-mediated immunity associated with secondary immunological deficiency diseases, for hyperlipoproteinemia and hypercholesteremia, and as adjuvants in diabetes therapy.

EP 0 555 618 A2

The present invention relates to dietetic and or pharmaceutical compositions containing lyophilized lactic bacteria which are qualitatively and quantitatively coordinated in an optimal manner for carrying out a re-balancing action on the intestinal flora, as well as a hypocholesterolemic and immunomodulating action. More specifically, the present invention relates to dietetic and or pharmaceutical compositions capable of normalizing the functions of the intestinal flora and promoting the body welfare, said mixture containing lyophilized lactic bacteria, an excipient and optionally a physiologically compatible drug.

In Bifidobacteria Microflora, Vol. 3(1), 29-33, 1984, the beneficial effect of administering Bifidobacterium to patients suffering from leukemia is described.

In FEMS Microbiology Reviews 46 (1987), 343-356, the therapeutical function of lactobacilli is disclosed, while Nobuo Suegara et al. (Microecology and Therapy, Vol. 15, 271-280 (1985)) state that oral administration of S. faecalis KAWAI greatly improves the lipid metabolism in human beings and animals.

From Microecology and Therapy, Vol. 14, 109-126 (1984) the effect of streptococcus cell extracts on hyperlipemia in rats, rabbits and human beings is known.

Other references describing the beneficial action of lactobacilli or other strains of activated bacilli are for example Bifidobacteria Microflora 1, 3-24, 1982 (Recent Trends in Research on Intestinal Flora), Microecology and Therapy 14, 267, 1984 (Intestinal Flora Associated Endotoxin), Microecology Therapy 16, 271-272, 1986 (Bifidobacterium bifidum Administration in Humans: a Controlled Clinical Study in Liver Cirrhosis), etc.

Finally in IT-A-1022625 food and farmaceutical compositions are described which perform an activity stimulating the production of gamma-interferon and contain lactobacilli S. thermophilus and L. bulgaricus.

However, in the cited literature no suggestion can be found on how to prepare compositions combining together several different lactic bacteria so as to exploit all their properties or synergistic effects, said compositions containing, in addition to said bacteria, usual adjuvants and pharmaceutically acceptable additives, eventually together with physiologically compatible drugs.

It is therefore an object of the present invention to provide an appropriate dietetic and/or pharmaceutical composition comprising lyophilized lactic bacteria qualitatively and quantitatively coordinated in an optimal manner for carrying out a re-balancing action on the intestinal flora, as well as a hypocholesterolemic and immunomodulating action, said composition containing, in addition to lyophilized lactic bacteria, also usual excipients or adjuvants, eventually together with some physiologically compatible drugs.

The present invention relates accordingly to dietetic and/or pharmaceutical compositions containing lyophilized lactic bacteria which are capable of promoting a welfare state in mammals, for example antagonizing the onset of diarrhoea, constipation, hypercholesteremia, liver disorders, immunosuppressant, enteritis, endotoxin absorption and production of endogenous toxic substances. It has been particularly found that the new pharmaceutical compositions object of the present invention are suitable for use in restoring the intestinal mucous membrane mass and modulating the epithelial cell kinetics and the enzyme potential of the intestine when they have been altered as a result of stress conditions. Other therapeutical suggestions for the embodiments of the invention comprise: gastroenteritis, colitis, constipation, diarrhoea, recolonization of the intestine after disorders due to foods, drugs, chemical or physical agents, after surgical interventions, chemotherapy or contagious deseases, hepatopathies resulting from toxic, metabolic or infectious causes, as an adjuvant for controlling hyperammonemia and endotoxinemia while liver insufficiency is in progress, in case of lack of humoral immunity and of mucosal and systemic cell-mediated immunity associated with secondary immunological deficiency diseases, hyperlipoproteinemia and hypercholesteremia, as an adjuvant in diabetes therapy.

The present invention further relates to dietetic and/or pharmaceutical compositions capable of normalizing the functions of the intestinal flora and promoting the body welfare, in that they promote the synthesis of vitamins and proteins, digestive, enzymatic and absorption processes, prevent colonization of pathogenic germs, stimulate the immune response and counteract arising of diarrhoea, constipation, enteritis, production of endogenous substances, absorption of endotoxins. These compositions find thus application in preventing and treating liver disorders, hypercholesteremia and immunosuppressant, and are characterized in that they contain several different lyophilized lactic bacteria together with pharmacologically acceptable excipients and optionally physiological compatible drugs.

The new lyophilized bacteria-containing compositions of the invention can be prepared by treating bacteria with appropriate technical expedients enabling the bacterium vitality to be recovered when they reach the digestive system. As a result, the administration of the compositions in question modifies the intestinal microflora, affects the expression of the membrane enzymes of microvilli and promotes the integrity of intestinal epithelial cells and related structures. The re-balancing action performed by the compositions of the invention in the intestine is followed by a reduction in the production of substances undesired by the proteolytic flora and a decreased absorption of endotoxins in the entire bodily system and a variation in the amount of cytokines produced by the intestinal wall and present in faeces. The synergism

of these actions induces an improvement in the clinical symptomatology and the laboratory parameters in subjects suffering from hepatopathies and trouble of the intestinal motility associated with dyspeptic-enterocolitis syndromes.

Bacteria present in the compositions object of the invention survive in the intestine, colonize it in a short time and stimulate the local and systemic immune apparatus. Studies about immunopharmacology have highlighted a development or normalization in the production of cytokines, among which interferons, interleukins and tumor necrosi factor (TNF) after ingestion of the compositions. By reactivating the humoral and cell-mediated immune system, the compositions can be used for the prophylaxis and therapy of immunological alterations associated with secondary immunological deficiency desease or stress situations.

Since some bacterial strains present in the compositions are capable of absorbing cholesterol, they also have a hypocholesterolemic and anti-atherosclerosis action. Another mechanism through which the compositions lower the chlolesterol levels in the blood is the stimulation of cholesterol excretion in faeces.

Bacteria or active ingredients that are inserted in the compositions of the invention are of the most different types: by way of example only we will mention lyophilized Bifidobacterium longum, lyophilized Bifidobacterium bifidum, lyophilized Bifidobacterium infantis, lyophilized L. acidophilus, lyophilized L. casei, lyophilized L. delbrueckii sub-species bylgaricus, lyophilized L. plantarum, lyophilized S. thermophilus and lyophilized S. faecium. Their concentration in the compositions of the invention ranges between 1.10^9 and 5.10^{12} bacteria per gram.

As further components, the compositions of the invention contain usual excipients currently employed for preparing pharmaceutical compositions in which normally the ratio of the active ingredient to the excipient ranges between 1:10 and 100:1.

If it is wished, the compositions may also contain the active ingredient alone.

The compositions of the invention can be made in the usual pharmaceutical forms known in literature, such as for example tablets, coated tablets, capsules, packets, solutions, suspensions, emulsions, suppositories, pellets, syrups, vaginal suppositories, ointments, creams and so on, and are prepared in the usual manner by mixing the active ingredients with excipients and/or carriers, optionally adding adjuvants and/or dispersing agents; should water be used as the diluent, also other organic solvents can be used in the form of adjuvants. Adjuvants can be for example: water, non-toxic organic solvents such as paraffines, vegetable oils (peanut oil or sesame oil) alcohols (ethanol, glycerine, for example), glycols (propylenglycol, polyethylenglycol), solid carriers such as for example natural mineral flours (kaolin, talc), synthetic mineral flours (silicates for example), sugar (cane sugar for example), emulsifiers (alkylsulfonates or arylsulfonates and the like), dispersers (lignin, methylcellulose, starch and polyvinylpyrrolidone, for example) and lubricants (magnesium stearate, talc, stearic acid, sodium laurylsolfonate, for example).

The administration takes place in the usual manner, preferably by oral route. In this case pharmaceutical forms adapted to this end can contain, in addition to usual excipients such as lactulose, dextrose, lactose, also additives such as sodium citrate, calcium carbonate, calcium dihydrogen phosphate, together with several additional substances such as starch, gelatin and the like. In case of liquid forms compatible colouring agents or flavoring substances may be added.

As an optional component, the compositions of the invention may contain a drug compatible with the bacteria used, capable of strengthening the activity of the active ingredients present therein. Among these drugs, the following can be mentioned: anticholinergic, antihistaminic, adrenergic, sedative, antiinflammatory, antipyretic, antiseptic, analgesic, antirheumatic, diuretic, antibacterial , hepatoprotector, antilipemic drugs, and so on.

Also active ingredients obtained from the bacterial walls or other bacterium cell components having a biological pro-host activity may be added.

For preparing the compositions, the individual microorganisms in the dehydrated form are mixed in appropriate proportions and the mixture is then added with the excipients and optionally the drug.

In one preferred embodiment of the invention, the dietetic and/or pharmaceutical composition comprises:

- 10 to 40% by weight of St. salivarium sub-species thermophilus (in the following S. thermophilus),
- 1 to 15% by weight of L. casei,
- 1 to 20% by weight of L. plantarum,
- 10 to 40% by weight of Bifidobatteri (as mixture),
- 1 to 15% by weight of L. acidophilus,
- 1 to 20% by weight of L. bulgaricus, and
- 1 to 20% by weight of S. faecium, with optionally 0 to 10% of an excipient and 0 to 20% of a compatible drug.

In a second preferred embodiment, the composition of the invention comprises eight lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to h) for the sake of simplicity) in the following amounts:

- 10 to 40% by weight of a),
- 2 to 18% by weight of b),
- 5 to 30% by weight of c),
- 1 to 15% by weight of d),
- 1 to 15% by weight of e)
- 1 to 20% by weight of f),
- 1 to 20% by weight of g), and
- 3 to 25% by weight of h), optionally with an excipient and a compatible drug in the above stated amounts.

In a third preferred embodiment of the invention the composition comprises seven lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to g) for the sake of simplicity) in the following amounts:

- 10 to 40% by weight of a),
- 2 to 18% by weight of b),
- 5 to 30% by weight of c),
- 2 to 30% by weight of d),
- 1 to 20% by weight of e),
- 1 to 20% by weight of f), and
- 3 to 25% by weight of g), optionally with an excipient and a compatible drug in the above stated amounts.

In a fourth preferred embodiment, the composition of the invention comprises six lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium, (identified by reference letters a) to f) for the sake of simplicity) in the following amounts:

- 10 to 40% by wieght of a),
- 2 to 18% by weight of b),
- 5 to 30% by weight of c),
- 2 to 30% by weight of d),
- 2 to 40% by weight of e), and
- 3 to 25% by weight of f), optionally with an excipient and a compatible drug in the above stated amounts.

In a fifth preferred embodiment of the invention, the composition comprises five lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to e) for the sake of simplicity) in the following amounts:

- 10 to 40% by weight of a),
- 2 to 18% by weight of b),
- 5 to 30% by weight of c),
- 4 to 70% by weight of d), and
- 3 to 25% by weight of e), optionally with an excipient and a compatible drug in the above stated amounts.

In a sixth preferred embodiment, the composition of the invention comprises four lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to d) for the sake of simplicity) in the following amounts:

- 10 to 40% by weight of a),
- 7 to 40% by weight of b),
- 4 to 70% by weight of c), and
- 3 to 30% by weight of d) optionally with an excipient and a compatible drug in the above stated amounts.

In a seventh preferred embodiment, the composition of the invention comprises three lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference

letters a) to c) for the sake of simplicity) in the following amounts:

- 10 to 40% by weight of a),
- 10 to 60% by weight of b), and
- 5 to 30% by weight of c), optionally with an excipient and a compatible drug in the above stated amounts.

In an eighth preferred embodiment, the composition of the invention comprises two lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) and b) for the sake of simplicity) in the following amounts:

- 10 to 90% by weight of a), and
- 90 to 10% by weight of b), optionally with an excipient and a compatible drug in the above stated amounts.

A most preferred composition of the invention comprises:

- 33% by weight of S. thermophilus,
- 9% by weight of L. casei,
- 10% by weight of L. plantarum,
- 9% by weight of L. acidophilus,
- 10% by weight of L. bulgaricus, and
- 29% by weight of a mixture of Bifidobatteri, optionally with 0 to 10% of an excipient and 0 to 20% of a compatible drug.

Both the individual strains and the final mixture have been submitted to microbiological tests for the purpose of evaluating both the number of microorganisms and possible pollutants. For counting of the bacteria forming the mixture, the following media have been employed:

Streptococci	M17 Agar - incubation over 48 hours
Lactobacilli and bifidobacteria	MRS Agar - incubation in anaerobiosis at 37 °C over 72 hours

For counting of possible pollutants the following media have been employed:

Coliiformes	deoxycholate agar -inoculation at 32 °C over 24 hours
Enterococci	KF agar + TTC incubation at 37 °C over 48 hours
Non-lactic flora	gel agar - incubation at 32 °C over 72 hours

Microbiological tests on the mixtures have given the following average results:

Coliiformes	< 100
Enterococci	absent/g
Non-lactic flora	absent/g

From the above results it is possible to conclude that, for the entirety of the microorganisms being part of the compositions, high charges have been achieved by virtue of the optimal conditions both of growth, and of concentration and drying up.

Pharmacological Part

Studies carried out on healthy volunteers or volunteers suffering from some of the above mentioned pathologies have proved the efficiency of the compositions in question.

Reproduced hereinafter are some of the parameters that have been measured according to the good usual medical procedure carried out in laboratory, that is: cholesterol, GOT, T CD4 + lymphocytes/CD8 + lymphocytes ratio, GPT, gamma GT and NK (natural killer) activity.

CHOLESTEROL (mg dl)				
	time 0	time 1	time 2	time 3
average	190.6	179.0	174,4	184.2
Maximum	285.3	264,8	247,5	267,4
Minimum	108,3	118.1	108,6	113,6
SD	59,9	44,7	44,0	54,3

GOT (IU/ml)				
	time 0	time 1	time 2	time 3
average	29.8	25,8	26,0	23,3
Maximum	97,3	88,1	81,5	59,1
Minimum	13,2	6,8	10,8	10,8
SD	23,1	22,0	19,1	13,7

CD4 TO CD8 RATIO				
	time 0	time 1	time 2	time 3
average	1,45	1,75	1,64	1,27
Maximum	2,71	4,10	4,32	2,76
Minimum	0,96	1,05	0,97	0,58
SD	0,57	0,87	0,95	0,60

GPT (IU/ml)				
	time 0	time 1	time 2	time 3
average	31,9	31,2	26,6	24,5
Maximum	131,6	132,6	99,1	91,0
Minimum	10,1	7,1	7,5	6,7
SD	34,5	35,4	26,3	24,1

gamma **GT** (IU/ml)				
	time 0	time 1	time 2	time 3
average	41,2	40,3	37,5	38,5
Maximum	127,0	137,8	129,1	125,6
Minimum	12,3	12,5	9,2	9,7
SD	34,3	37,1	35,4	34,3

NK Activity (12.5:1)				
	time 0	time 1	time 2	time 3
average	44,0	67,5	49,9	41,0
Maximum	62,0	97,0	62,0	56,0
Minimum	24.0	47,0	25,0	14,0
SD	12,2	16,9	11,4	14,1

NK Activity (25:1)				
	time 0	time 1	time 2	time 3
average	56,4	80,5	54,6	48,1
Maximum	66,0	100,0	69,0	63,0
Minimum	42,0	51,0	37,0	23,0
SD	9,8	13,3	9,5	12,0

NK Activity (50:1)				
	time 0	time 1	time 2	time 3
average	60,8	87,8	59,1	51,8
Maximum	74,0	100,0	65,0	63,0
Minimum	48,0	69,0	42,0	30,0
SD	7,5	10,5	6,9	11,1

EXAMPLE

Strains used in the following formulation, given by way of example only, are as follows:

- thermophile Streptococci consist of a mixture of two strains from a yogurt culture and a starter used for preparation of cheeses;
- Lactobacillus bulgaricus is represented by a strain isolated from a yogurt culture;
- Lactobacillus acidophilus is present in a mixture consisting of two strains of human origin isolated from a special yogurt;
- Bifidobacteria come from the intestinal flora of newborn babies;
- Lactobacillus casei has been isolated from a culture employed in the production of cheeses, and
- Lactobacillus plantarum has been isolated from vegetables in progress of fermentation.

In order to obviate problems due to possible phage attacks, these strains can obviously be replaced by other cultures having the same features and origins, but provided with a different phage sensitiveness.

Culture Preparation

The individual strains maintained in a lyophilized and frozen form have been grown in synthetic media specific for each species. The fundamental component of the culture medium is permeate obtained by ultrafiltration of serum or milk, to which minima amounts of biological activators are added depending on the species. After sterilization, the culture medium is inoculated with a strain per species or 1 to 3 strains belonging to the same genotype. Cultures have been incubated upon determination of optimal parameters for each strain: temperature, time, pH values and stirring.

Industrial cultures have been concentrated by centrifugation, and lyophilization has been then carried out according to standard methodologies.

After lyophilisation the cell mass has been pulverized under sterile conditions. The individual cultures submitted to chemical and microbiological tests have been maintained at 5 °C in hermetic vessels.

Preparation of the individual species

1) Streptococcus salivarius sub-species thermophilus

- Mother

The mother has been prepared by inoculating the strain in a medium consisting of 5% of permeate + 1% of yeast extract and incubated at 44 °C over 3 hours.

- Medium

Permeate	5%
Yeast extract	1%

- Fermentation parameters

Fermenter:	72 l Applikon
Percent of inoculation:	1%
Incubation temperature	44 °C
Stirring speed	160 rpm
Neutralization set point	pH = 6.00
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	3h 30m
Final cooling:	24 °C

- Concentration parameters

Centrifuge type:	Westfalia SA1
Centrifugation temperature:	24 °C
flow rate:	24 l/h
(The concentrate has been then centrifuged again using a laboratory centrifuge at 6000 rpm over 20 minutes).	

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400
Lyophilization protector:	a solution of lactose

Results: the number of microorganisms during the different steps of the process are reproduced in the following Table:

Steps	U.F.C./g
End of fermentation	2.4E9
Concentrate	1.6E11
Lyophilized	7.4E11
U.F.C. = Colony-forming units E9 = one thousand millions E11 = one hundred thousand millions	

No particular problems have been, found in the preparation of this microorganism. Therefore the cell loss during the different steps of the process could be greatly limited and a high bacterial charge could be achieved in the lyophilized product.

2) Lactobacillus plantarum

- Mother

Prepared in MRS culture medium and incubated at 33 °C over 5 hours.

- Medium

Permeate	5%
Yeast extract	1%
Glucose	2.5%

- Fermentation parameters

Fermenter:	Applikon
Percent of inoculation:	1%
Incubation temperature	33 °C
Stirring speed	110 rpm
Neutralization set point	pH = 6.00
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	15 h

Cell inactivation after fermentation by pasteurization at 80 °C over 15 minutes.

- Concentration parameters

Centrifuge type:	Westfalia SA1
Centrifugation temperature:	60 °C
flow rate:	40 l/h

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400
Lyophilization protector:	a solution of lactose

The number of microorganisms during the different process steps is reproduced in the following Table:

Steps	U.F.C./g	Count g in Thoma
End of fermentation	9.2E8	-
Lyophilized	-	1.0E11
U.F.C. = Colony-forming units E8 = one hundred millions E11 = one hundred thousand millions.		

3) Lactobacillus casei

- Mother

Prepared in MRS culture medium and incubated at 37 °C over 8 hours and 30 minutes

- Medium

Permeate	5%
Yeast extract	1%
Glucose	1%

- Fermentation parameters

Fermenter:	Applikon
Inoculation percent:	1%
Incubation temperature	37 °C
Stirring speed	110 rpm
Neutralization set point	pH = 5.40
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	15 h

- Concentration parameters

Centrifuge type:	Westfalia SA1
Centrifugation temperature:	60 °C
Flow rate:	46 l/h

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400
Lyophilization protector:	a lactose solut.

Results: the number of microorganisms during the different process steps is reproduced in the following Table:

Steps	U.F.C./g	Count/g in Thoma
End of fermentation	1.0E9	-
Lyophilized	-	1.0E11
U.F.C. = Colony-forming units E8 = one thousand millions E11 = one hundred thousand millions.		

4) Mixture of bifidobacteria (Bifidobacterium infantis -Bifidobacterium longum - Bifidobacterium breve)

- Mother

The mother has been prepared by inoculating the strains in a medium consisting of 10% of powdered skimmed milk + 0.5% of glucose + 1% of yeast extract and incubated at 38 °C over 15 hours.

- Medium

Permeate	4%
Yeast extract	1%
Bacto Soytone	0.25%
Glucose	0.5%

- Fermentation parameters

Fermenter:	Applikon
Inoculation percent:	2%
Incubation temperature	38 °C
Stirring speed	110 rpm
Neutralization set point	pH = 6.00
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	15 h
Cooling at the end of fermentation:	24 °C

- Concentration parameters

Centrifuge type:	Westfalia SA1
Centrifugation temperature:	24 °C
Flow rate:	42 l h
(The obtained concentrate has been then centrifuged again with a laboratory centrifuge at 6000 rpm over 20 minutes).	

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400

Lyophilization protector: a solution of powdered slimmed milk + yeast extract + lactose + sodium maleate has been prepared.

Results: the number of microorganisms during the different process steps is reproduced in the following Table:

Steps	U.F.C./g
End of fermentation	1.7E9
Concentrate	7.0E10
Lyophilized	3.8E11
U.F.C. = Colony-forming units E9 = one thousand millions E10 = ten thousand millions E11 = one hundred thousand millions.	

In this case too, in which bacteria are considered of "hard" growing, no particular problems have been found during the different preparation steps and the number of microorganisms is high both in the fermentation and on the lyophilized.

5) Lactobacillus acidophilus

- Mother

Prepared in a medium consisting of 5% of permeate + 1% of yeast extract + 1% of glucose + 1% of Tween (Registered Trademark) 80 and incubated at 37 °C over 15 hours.

- Medium

Permeate	5%
Yeast extract	1%
Glucose	1%
Tween (Registered Trademark) 80	0.1%

- Fermentation parameters

Fermenter:	Applikon
Inoculation percent:	1%
Incubation temperature	37 °C
Stirring speed	110 rpm
Neutralization set point	pH = 6.00
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	15 h
Cooling at the end of fermentation:	24 °C

- Concentration parameters

Centrifuge type:	Westfalia SA1
Centrifugation temperature:	24 °C
Flow rate:	42 l/h
(The obtained concentrate has been then centrifuged again with a laboratory centrifuge at 6000 rpm over 20 minutes).	

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400

Lyophilization protector: a solution of lactose and anhydrous mixture consisting of powdered skimmed milk + yeast extract + lactose + sodium glutamate + Tween (Registered Trademark) 80.

Results: the number of microorganisms during the different process steps is reproduced in the following Table:

Steps	U.F.C./g
End of fermentation	2.9E8
Concentrate	2.3E10
Lyophilized	2.0E10
U.F.C. = Colony-forming units E8 = one hundred millions E10 = ten thousand millions	

6) Lactobacillus delbrueckii sub-species bulgaricus

- Mother

Prepared in a medium consisting of 5% of permeate + 1% of yeast extract + 1% of beef extract + 1% of glucose + 0.1% of Tween (Registered Trademark) 80 and incubated at 44 °C over 4 hours and 30 minutes.

- Medium

Permeate	5%
Yeast extract	1%
Beef extract	1%
Glucose	1%
Tween (Registered Trademark) 80	0.1%

- Fermentation parameters

Fermenter:	Applikon
Inoculation percent:	1%
Incubation temperature	44 ° C
Stirring speed	110 rpm
Neutralization set point	pH = 5.60
Neutralizing substance type:	ammonium hydrate (sol to 10%)
Fermentation time:	7 h
Cooling at the end of fermentation:	24 ° C

- Concentration parameters

Installation: pilot unit for Hydro Air Research microfiltration with two serial ceramic diaphragms each having a 0.2 m^2 filtrating surface.

Microfiltration temperature	30 ° C
Operating conditions	recirculation flow rate 4000 l/h input pressure 2.7 bar output pressure 1.2 bar average flow of the permeate 30 l/h x m^2
(The obtained concentrate has been then centrifuged again with a laboratory centrifuge at 6000 rpm over 20 minutes).	

- Lyophilization

Lyophilizer	Edwards MINI -FAST 3400

Lyophilization protector: a solution of lactose and anhydrous mixture consisting of powdered skimmed milk + yeast extract + lactose + sodium glutamate + Tween (Registered Trademark) 80.

Results: the number of microorganisms during the different process steps is reproduced in the following Table:

Steps	U.F.C./g
End of fermentation	2.9E9
Concentrate	2.4E10
Lyophilized	3.5E9
U.F.C. = Colony-forming units E9 = one thousand millions E10 = ten thousand millions	

Claims

1. Dietetic and/or pharmaceutical compositions capable of normalizing the functions of the intestinal flora and of promoting the body welfare in human beings and animals, characterized in that they contain several different lyophilized lactic bacteria in a concentration of from 1.10^9 to 5.10^{12} bacteria per gram together with pharmacologically acceptable excipients and optionaly physiological compatible drugs, said bacteria being selected from lyophilized Bifidobacterium longum, lyophilized Bifidobacterium bifidum, lyophilized Bifidobacterium infantis, lyophilized L. acidophilus, lyophilized L. casei, lyophilized L. delbrueckii sub-species bylgaricus, lyophilized L. plantarum, lyophilized S. thermophilus and lyophilized S. faecium and in that they can also contain active ingredients obtained from the bacterial walls or other bacterium cell components having a biological pro-host activity.

2. A composition according to claim 1, characterized in that it comprises:
   - 10 to 40% by weight of S. thermophilus,
   - 1 to 15% by weight of L. casei,
   - 1 to 20% by weight of L. plantarum,
   - 10 to 40% by weight of Bifidobatteri (as a mixture),
   - 1 to 15% by weight of L. acidophilus,
   - 1 to 20% by weight of L. bulgaricus, and
   - 1 to 20% by weight of S. faecium, with optionally 0 to 10% of an excipient and 0 to 20% of a compatible drug.

3. A composition according to claims 1 and 2, characterized in that it comprises eight lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to h) for the sake of simplicity) in the following amounts:
   - 10 to 40% by weight of a),
   - 2 to 18% by weight of b),
   - 5 to 30% by weight of c),
   - 1 to 15% by weight of d),
   - 1 to 15% by weight of e)
   - 1 to 20% by weight of f),
   - 1 to 20% by weight of g), and
   - 3 to 25% by weight of h), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

4. A composition according to claims 1 and 2, characterized in that it comprises seven lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to g) for the sake of simplicity) in the following amounts:
   - 10 to 40% by weight of a),
   - 2 to 18% by weight of b),
   - 5 to 30% by weight of c),
   - 2 to 30% by weight of d),
   - 1 to 20% by weight of e),
   - 1 to 20% by weight of f), and

- 3 to 25% by weight of g), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

5. A composition according to claims 1 and 2, characterized in that it comprises six lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium, (identified by reference letters a) to f) for the sake of simplicity) in the following amounts:
   - 10 to 40% by wieght of a),
   - 2 to 18% by weight of b),
   - 5 to 30% by weight of c),
   - 2 to 30% by weight of d),
   - 2 to 40% by weight of e), and
   - 3 to 25% by weight of f), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

6. A composition according to claims 1 and 2, characterized in that it comprises five lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to e) for the sake of simplicity) in the following amounts:
   - 10 to 40% by weight of a),
   - 2 to 18% by weight of b),
   - 5 to 30% by weight of c),
   - 4 to 70% by weight of d), and
   - 3 to 25% by weight of e), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

7. A composition according to claims 1 and 2, characterized in that it comprises four lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to d) for the sake of simplicity) in the following amounts:
   - 10 to 40% by weight of a),
   - 7 to 40% by weight of b),
   - 4 to 70% by weight of c), and
   - 3 to 30% by weight of d), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

8. A composition according to claims 1 and 2, characterized in that it comprises three lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) to c) for the sake of simplicity) in the following amounts:
   - 10 to 40% by weight of a),
   - 10 to 60% by weight of b), and
   - 5 to 30% by weight of c), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

9. A composition according to claims 1 and 2, characterized in that it comprises two lyophilized lactic bacteria selected from Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus and S. faecium (identified by reference letters a) and b) for the sake of simplicity) in the following amounts:
   - 10 to 90% by weight of a), and
   - 90 to 10% by weight of b), optionally with an excipient and a compatible drug in the amounts stated in claim 2.

10. A composition according to claims 1 and 2, characterized in that it comprises:
   - 33% by weight of S. thermophilus,
   - 9% by weight of L. casei,
   - 10% by weight of L. plantarum,
   - 29% by weight of a mixture of Bifidobatteri,

- 9% by weight of L. acidophilus,
- 10% by weight of L. bulgaricus, optionally with 0 to 10% of an excipient and 0 to 20% of a compatible drug.

11. A composition according to anyone of the preceding claims, characterized in that drugs are selected from anticonvulsant, anticholinergic, antihistaminic, adrenergic, sedative, antiinflammatory, antipyretic, antiseptic, analgesic, antirheumatic, diuretic, antipsychotic, antibacterial, and analeptic drugs.

12. A composition according to anyone of the preceding claims, characterized in that the ratio of the active ingredient to the excipient is in the range of 1:10 to 100:1.

13. A composition according to anyone of the preceding claims, characterized in that it is in the form of tablets, coated tablets, capsules, packets, solutions, emulsions, suspensions, pellets, suppositories, syrups, vaginal suppositories, ointments or creams.

14. A process for preparing dietetic and/or pharmaceutical compositions according to claims 1 to 13, characterized in that the individual microorganisms in a dehydrated form are mixed, added with an excipient and optionally with an appropriate drug, and the final mixture is poured into vials which are sealed and stored.

15. Use of the dietetic and/or pharmaceutical compositions according to claims 1 to 13 for promoting the synthesis of vitamins and proteins, digestive, enzymatic and absorption processes, preventing colonization of pathogenic germs, modulating the local and systemic immune response and antagonizing the onset of diarrhoea, constipation, enteritis, production of toxic endogenous substances, absorption of endotoxins, liver disorders, hypercholesteremia and immunosuppressant, restoring the intestinal mucous membrane mass and modulating the epithelial cell kinetics and the enzyme potential of the intestine when they have been altered as a result of stress conditions, recolonizing the intestine after disorders due to foods, drugs, chemical or physical agents, after surgical operations, chemoterapy or contagious deseases, treating hepatopathies resulting from toxic, metabolic or infectious causes.

16. Use of the dietetic and/or pharmaceutical composizions according to claims 1 to 13 as adjuvants for controlling hyperammonemia and endotoxinemia while liver insufficiency is in progress, in case of lack of humoral immunity associated with secondary immunological deficiency diseases, hyperlipoproteinemia and as adjuvants in diabetes therapy.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 555 618 A3**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **92830653.9**

(51) Int. Cl.[5] **A23L 1/03**, A61K 35/74

(22) Date of filing: **04.12.92**

(30) Priority: **10.02.92 IT MI920256**

(43) Date of publication of application:
18.08.93 Bulletin 93/33

(84) Designated Contracting States:
BE CH DE DK ES FR GB GR IE LI LU NL PT

(88) Date of deferred publication of the search report:
22.06.94 Bulletin 94/25

(71) Applicant: **Cavaliere Vesely, Renata Maria Anna**
Via S. Orsola 11
I-20123 Milano(IT)
Applicant: **De Simone, Claudio**
Via Nuoro 10
I-00040 Ardea (Roma)(IT)

(72) Inventor: **Cavaliere Vesely, Renata Maria Anna**
Via S. Orsola 11
I-20123 Milano(IT)
Inventor: **De Simone, Claudio**
Via Nuoro 10
I-00040 Ardea (Roma)(IT)

(74) Representative: **Righetti, Giuseppe**
Bugnion S.p.A.
Via Carlo Farini, 81
I-20159 Milano (IT)

(54) Dietetic and/or pharmaceutical compositions containing lyophilized lactic bacteria, their preparation and use.

(57) Pharmaceutical compositions containing highly-concentrated lyophilized lactic bacteria in combination with pharmacologically acceptable excipients and optionally suitable physiologically compatible drugs, are adapted for restoring the intestinal mucous membrane mass and modulating the epithelial cell kinetics and the enzyme potential of the intestine when they have been altered as a result of stress conditions, and in addition in case of gastroenteritis, colitis, constipation, diarrhoea, for recolonizing the intestine after food disorders, surgical interventions, chemotherapy or contagious deseases, hepatopathies resulting from toxic, metabolic or infectious causes, as adjuvants for controlling hyperammonemia and endotoxinemia while liver insufficiency is in progress, in case of lack of humoral immunity and of mucosal and systemic cell-mediated immunity associated with secondary immunological deficiency diseases, for hyperlipoproteinemia and hypercholesteremia, and as adjuvants in diabetes therapy.

EP 0 555 618 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 92 83 0653

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X A	US-A-4 806 368 (M.S.REDDY) * column 1, line 10 - line 20; claims * ---	1,11-16 2-10	A23L1/03 A61K35/74
X A	EP-A-0 291 578 (KOPALNIA SOLI "WIELICKA") * column 2, line 48 - column 3, line 8; claims 1,4; example III * ---	1,11-16 2-10	
X	DATABASE WPI Section Ch, Week 8916, Derwent Publications Ltd., London, GB; Class A96, AN 89-119459 & JP-A-1 066 124 (FREUND SANGYO) 13 March 1989 * abstract * ---	1,11-16	
X	PATENT ABSTRACTS OF JAPAN vol. 9, no. 309 (C-318)5 December 1985 & JP-A-60 149 527 (SEIKENKAI) 7 August 1985 * abstract * ---	1,11-16	
P,X	US-A-5 143 845 (T.MASUDA) * column 7, line 15 - line 23; claims * & JP-A-63 063 620 (...) 22 March 1988 & JP-A-63 091 076 (...) 21 April 1988 ---	1,11-16	
X A	EP-A-0 228 861 (ADVANCE) * claims * * example 5 * ---	1,11-16 2-10	
D,A	MICROECOLOGY AND THERAPY vol. 14 , 1984 , HERBORN-DILL pages 109 - 126 Y.KAWAI 'Effect of cellular extracts of streptococci on hyperlipidemia in rats,rabbits and humans' * the whole document * ---	1-16	
	-/--		

TECHNICAL FIELDS SEARCHED (Int.Cl.5)
A23L
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27 April 1994	Van Moer, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 92 83 0653

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,A	BIFIDOBACTERIA MICROFLORA vol. 3, no. 1 , 1984 pages 29 - 33 T.KAGEYAMA ET AL. 'The effect of bifidobacterium administration in patients with leukemia' * the whole document * ---	1-16	
D,A	BIFIDOBACTERIA MICROFLORA vol. 1, no. 1 , 1982 pages 3 - 24 T.MITSUOKA 'Recent trends in research on intestinal flora' * the whole document * ---	1-16	
D,A	MICROECOLOGY AND THERAPY vol. 16 , 1986 , HERBORN-DILL pages 271 - 272 D.MÜTING ET AL. 'Bifidobacterium bifidus administration in humans:a controlled clinical study in liver cirrhosis' * the whole document * ---	1-16	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
D,A	MICROECOLOGY AND THERAPY vol. 15 , 1985 , HERBORN-DILL pages 271 - 280 N.SUEGARA ET AL. 'Hypolipidemic effect of streptococcus faecalis kawai in humans and mechanisms of serum lipid reduction' * the whole document * -----	1-16	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27 April 1994	Van Moer, A

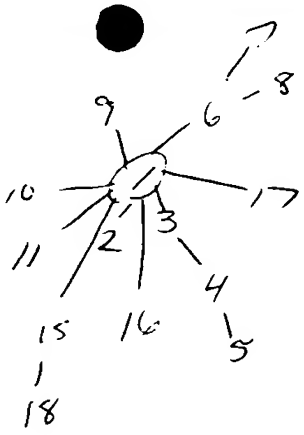
CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)


9
6
8
10
17
1
3
11
2
4
15
16
5
18


12
13
14